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Development of a real-time PCR Assay for the Detection of Egg Drop Syndrome 1976 Virus

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1. Summary

1.1. Summary

The Egg drop syndrome 1976 virus (EDSV 1976), also named Duck adenovirus 1 (DAdV-1), belongs to the genus *Atadenovirus* within the family *Adenoviridae*. Infection of laying flocks with EDSV 1976 leads to a reduced egg production and changes in eggshell quality including thin, rough or soft, or even absent shells. Current diagnostic tests include virus isolation, conventional polymerase chain reaction (PCR) or serological methods. In order to establish a more sensitive and less time consuming assay, a real-time quantitative polymerase chain reaction (qPCR) was developed. Primers and probe within the open reading frame of EDSV 1976 endoprotease gene (L3 23K) were designed. The sensitivity was tested using 10-fold dilution series of a plasmid containing the amplified PCR fragment. The assay was able to detect 30 copies in 100% of the experiments and even 3 copies were amplified in 75%. Specificity was determined testing DNA of several viral and bacterial avian pathogens. All samples tested yielded a negative result with the exception of DNA from EDSV 1976. The new method was subsequently applied to screen Swiss laying flocks with a decreased egg production for the presence of EDSV 1976. Virus could not be detected in any of the 8 herds tested. In summary, the newly established real-time PCR is both sensitive and specific, and represents a reliable method for the diagnosis of Egg drop syndrome 1976.

Keywords: Egg drop syndrome 1976 virus, real-time PCR assay, Swiss layers

1.2. Zusammenfassung

Das Egg drop Syndrom 1976 Virus (EDSV 1976), welches auch Duck Adenovirus 1 genannt wird (DAdV-1), wird dem Genus Atadenovirus innerhalb der Familie Adenoviridae

zugeordnet. Eine Infektion von Legehennen mit EDSV 1976 führt zu einer reduzierten Eiproduktion sowie zu Veränderungen der Eischalenqualität, was sich in dünnen, rauen, weichen oder sogar fehlenden Eischalen äussert. Heutzutage wird die Diagnose mittels Virusisolation, konventioneller Polymerase Kettenreaktion (PCR) oder Serologie gestellt. Um einen sensitiveren und schnelleren Test zu entwickeln, wurde eine real-time quantitative Polymerase Kettenreaktion (qPCR) etabliert. Die Primer und Sonden zum Nachweis von EDSV 1976 DNA wurden innerhalb des Open Reading Frame der Endoprotease (L3 23K) von EDSV 1976 konstruiert. Die Sensitivität wurde mithilfe einer Verdünnungsreihe eines Plasmides, welches das mit der PCR amplifizierte Fragment enthält, bestimmt. 30 Plasmidkopien konnten in 100% der Ansätze nachgewiesen werden, während in 75% der Fälle sogar 3 Plasmidkopien amplifiziert werden konnten. Die Spezifität wurde getestet, indem DNA von verschiedenen viralen und bakteriellen aviären Pathogenen eingesetzt wurde. Abgesehen von EDSV 1976 konnte keine andere DNA amplifiziert werden. Ferner wurde die neu entwickelte Methode angewendet, um Schweizer Legeherden mit reduzierter Eiproduktion auf das Vorkommen von EDSV 1976 zu testen. In keiner der untersuchten 8 Herden konnte das Virus nachgewiesen werden. Zusammenfassend kann gesagt werden, dass die neu entwickelte real-time PCR sowohl sensitiv als auch spezifisch ist und eine verlässliche Methode zur Diagnose von Egg drop Syndrom 1976 darstellt.

Stichworte: Egg drop Syndrom 1976 Virus, real-time PCR, Schweizer Legehennen

2. Introduction

Egg drop syndrome 1976 (EDS 76) is an economically important viral disease of chickens and quails. The disease is characterized by a sudden decrease in laying performance as well as changes in eggshell quality (Senthilkumar *et al*., 2004; CFSPH Egg drop syndrome factsheet, 2006). Causative agent of EDS 76 is an Atadenovirus assigned to the family Adenoviridae (Fitzgerald, 2008). This family consists of five serologically distinguishable genera: Mastadenoviruses, Aviadenoviruses, Siadenoviruses, Ichtadenoviruses as well as Atadenoviruses (Table 1). Adenoviruses are characterized by a linear double-stranded DNA genome and an icosahedral structure without an envelope (Kaaden, 2002). The capsides are composed of 12 penton and 240 hexon proteins. The Adenovirus genome has a size of 36'000 to 38'000 base pairs (bp), depending on the virus type. The genome consists of 5 coding regions, from which 4 are transcribed early during infection (E1-E4). Genes encoding for structural proteins are transcribed late (L). A mature late primary transcript is processed into 5 messenger RNAs (L1-L5). Adenoviruses proliferate within the nucleus, where initially transcription of the early genes and synthesis of the early proteins occurs. In a next step, the DNA replicates itself, following transcription of the late genes and synthesis of the late proteins. Finally, the virions are assembled (Modrow *et al*., 2003).

Avian adenoviruses are widespread pathogens in fowl and wild birds. Most avian adenoviruses proliferate without causing any clinical symptoms, but often figure as opportunists in case of previously existing diseases. However, there are some specimens that cause specific diseases. Avian adenoviruses are distributed over three different genera within the family of Adenoviridae: Aviadenovirus (group 1 avian adenoviruses), Siadenovirus (group 2 avian adenoviruses) and Atadenovirus (group 3 avian adenoviruses) (Fitzgerald, 2008). Today, Aviadenoviruses are segregated into 7 species, Fowl adenovirus A through E, Falcon adenovirus A and Goose adenovirus (Table 1). Relevant pathogenic agents within the

genus Aviadenovirus are Fowl adenovirus 1 (FAdV-1), which is classified into the species Fowl adenovirus A and causes quail bronchitis, and Fowl adenovirus 4 (FAdV-4), member of Fowl adenovirus C, causative organism involved in hydropericardium syndrome (McConnell Adair & Fitzgerald, 2008; VMRI based on ICTV decisions, 2009). However, Siadenoviruses can only be divided into 3 species, Frog adenovirus, Raptor adenovirus A and Turkey adenovirus A, which causes hemorrhagic enteritis in turkeys, marble spleen disease in pheasants and splenomegaly in chickens (Fitzgerald, 2008).

Atadenoviruses are classified into 5 species: Duck adenovirus A, Snake adenovirus A, Ovine adenovirus D, Bovine adenovirus D and Possum adenovirus (Table 1). The species Duck adenovirus A includes one single member, Duck adenovirus 1 (DAdV-1), which is the official name of the Egg drop syndrome 1976 virus (VMRI based on ICTV decisions, 2009). The size of EDSV 1976 ranges from 76 to 80 nm. EDSV 1976 differs in its morphology from other adenoviruses by having only one fiber per penton base (McConnell Adair & Smyth, 2008). Like many other Adenoviruses, EDSV 1976 is able to agglutinate erythrocytes (Monreal, 1992). Erythrocytes of chickens, ducks, geese, turkeys, pigeons and peacocks are agglutinated by EDSV 1976, in contrast to mammalian erythrocytes. There is only one serotype of EDSV 1976, but three different genotypes can be distinguished on the basis of restriction enzyme digestion (Mc Connell Adair & Smyth, 2008). The EDSV 1976 genome contains 33’213 base pairs and has a molecular weight of 21.9 x 10^6 d (Hess *et al.,* 1997), which is slightly smaller than the genome of FAdV-1 (28.9 x 10^6 d) (Mc Connell Adair & Smyth, 2008).

Egg drop syndrome 1976 was first reported in the Netherlands in 1976, when adenoviruses were isolated from laying chickens. Since then, EDSV 1976 has been detected in chickens in Europe either serologically or by virus isolation in Belgium, France, Hungary, Great Britain, Italy, Northern Ireland and Denmark. The virus was also found in chickens in Israel, China, India, Japan, Singapore, Taiwan, New Zealand, Australia, South Africa, Nigeria, Brazil and

Mexico (McConnell Adair & Smyth, 2008). Except for some herds imported from France, EDS 76 has not been detected in Switzerland yet (Hoop, personal communication, 2010).

Although Egg drop syndrome 1976 has been detected mainly in chickens, it is assumed that ducks and geese are the natural hosts for the virus (Monreal, 1992; McConnell Adair & Smyth, 2008). However, it is supposed that virus transmission did not happen naturally, but virus had been transferred to chickens by a life vaccine, based on duck embryo cells (Monreal, 1992). Serological investigations demonstrate that EDSV 1976 also appears in mergansers, coots, grebes, cattle egrets, herring gulls, owls, storks, swans, guinea fowl and pigeons (CFSPH Egg drop syndrome factsheet, 2006; McConnell Adair & Smyth, 2008). Disease outbreaks have been reported from chickens, geese and quails (CFSPH Egg drop syndrome factsheet, 2006). EDSV 1976 was also isolated from healthy and diseased ducks, but experimental reproduction of the disease was not possible (McConnell Adair & Smyth, 2008). Turkeys can be infected under experimental conditions, but normally do not show clinical symptoms (McNulty & Smyth, 2002). However, a natural infection of turkeys apparently took place in Croatia in 2007 (Bidin *et al*., 2007). Vertical transmission from hen to egg is the most important source of infection and is followed by horizontal spread via faeces within the flock, resulting in oral uptake of the virus (Hess & Monreal, 2005). After experimental oral infection of chickens, EDSV 1976 reproduces in the nasal mucosa and then spreads through viremia into the lymphoid tissue, followed by affection of the oviduct (McConnell Adair & Smyth, 2008). Faeces can be contaminated by secretions of the oviduct and display a possible source of infection (McNulty & Smyth, 2002). Viral colonization of the uterine glands leads to dysfunction of eggshell generation (Hess & Monreal, 2005). Egg drop syndrome 1976 can manifest in three different forms: The classical type mainly occurs in breeders, where virus is first transmitted vertically, followed by horizontal transmission as described above (McNulty & Smyth, 2002; Hess & Monreal, 2005; McConnell Adair & Smyth, 2008). Horizontal transmission between flocks due to contaminated eggs can occur

and lead to an endemic type of the disease. The sporadic form of EDS 76 outbreaks results from water contaminated by duck, goose or wild bird faeces (McNulty & Smyth, 2002; McConnell Adair & Smyth, 2008).

Infection can occur at any age, and clinical signs can appear either at the beginning or at the top of egg production (Senthilkumar *et al*., 2004). EDSV 1976 induces decreased laying production and deficient eggshells and therefore is of great economical relevance in poultry (Dhinakar Raj *et al*., 2001; Dhinakar Raj *et al*., 2003; Senthilkumar *et al*., 2004). Eggs first show changes in shell colour, later thin-shelled or shell-less eggs occur. Furthermore, eggs with rough and sandpaperlike surface are produced. The rate of abnormal eggshells can amount up to 20%, and often a decrease in laying performance up to 50% appears. Individuals normally do not show many symptoms. Rarely, diarrhoea is observed (Hess & Monreal, 2005). In one case, respiratory disease was reported from goslings in Hungary (Ivanics *et al*., 2001).

Diagnosis of infection with EDSV 1976 is made by virus isolation or serological methods, such as haemagglutination inhibition test and ELISA (McNulty & Smyth, 2002). Compared with serology, virus isolation is not only less sensitive and less specific, but also more time-consuming. In addition, virus isolation depends on the presence of infectious virus particles. The presence of antibodies against EDSV 1976 indicates a previous infection, but there is no information whether the virus is still circulating in the host. Today, PCR is used as well for the detection of EDSV 1976 (Dhinakar Raj *et al*., 2001; Dhinakar Raj *et al*., 2003; Kumar *et al*., 2003). The advantages of molecular biology based over serological methods and virus isolation include a higher sensitivity and specificity as well as the fact that no infectious virus is needed (Büttner, 2002). For laboratory diagnostics, real-time PCR has several advantages over conventional PCR methods including a lower risk of contamination, the possibility of easier quantification and saving of time (Callison *et al*., 2006).

In order to establish a reliable, sensitive and specific test for the detection of EDSV 1976 in chicken samples we developed a TaqMan based quantitative real-time PCR assay (qPCR) amplifying a 145 bp fragment within the endoprotease gene region. In addition, samples collected from Swiss laying flocks showing loss in egg production were screened.

3. Materials and Methods

3.1. Sample collection

A total of 162 tissue samples were taken from 27 animals originating from 8 laying flocks experiencing problems in laying performance (Table 2). Chickens were dissected, and tissue samples removed from liver, infundibulum and magnum of the oviduct, trachea, kidney and lung were frozen at -80°C.

3.2. Viral and bacterial strains

EDSV 1976 strain 127 was used as the reference strain in order to establish our real-time PCR assay. Furthermore, DNA from Bovine adenovirus 1 (BAdV-1), Fowl adenovirus 1 (FAdV-1), Gallid herpesvirus 1 (GaHV-1), Gallid herpesvirus 2 (GaHV-2), Mycoplasma synoviae, Mycoplasma meleagridis and Mycoplasma gallisepticum was tested. All organisms used in this study are listed in Table 3.

3.3. Nucleic acid extraction

3.3.1. DNA extraction from tissue samples

DNA was extracted from tissue samples using the DNeasy Blood and Tissue Kit (QIAgen, Hilden, Germany). Tissue samples were cut into small pieces up to 25 mg and digested with 180 µl ATL buffer and 20 µl Proteinase K for 3-5 hours or overnight shaking at 56°C. In order to verify the extraction procedure with regard to contamination a control sample consisting only of reagents was used and treated the same way. After a short centrifugation step, 200 µl AL buffer was added and the sample was properly mixed. Subsequently, the mixture was incubated shaking at 70°C for 10-20 minutes and following centrifugation loaded

on a QIAshredder Mini Spin Column to avoid plugging. 200 µl ethanol (100%) was added to the filtrate and the mixture was pipetted onto a QIAamp Mini Spin Column. After centrifugation at 8000 rpm for 1 minute the accumulated filtrate was discarded and the column was placed on a new collection tube. The column was washed using buffers AW1 and AW2 according to the manufacturer's protocol. Finally, DNA was eluted in 100 µl AE buffer and stored at -20°C.

3.3.2. DNA extraction from viruses and bacteria

DNA was extracted from EDSV 1976, BAdV-1, FAdV-1, GaHV-1, GaHV-2, Mycoplasma synoviae, Mycoplasma meleagridis and Mycoplasma gallisepticum in order to test the specificity. DNA extraction was performed using the QIAgen DNeasy Blood and Tissue Kit (QIAgen, Hilden, Germany). 100 µl of virus suspension or 50 µl of bacteria suspension and 50 µl of water was digested with 180 µl ATL buffer and 20 µl Proteinase K for 3-5 hours. The further extraction was carried out in the same way as the extraction from tissue samples, DNA was eluted in 100 µl AE buffer and stored at -20°C.

3.4. Conventional PCR

Primer and probe sequences (Table 4) were designed with Applied Biosystems Primer Express Software version 3.0.® (Applied Biosystems, Foster City, CA, USA) following the manufacturer guidelines. Primers to detect EDSV 1976 were designed based on the gene encoding the endoprotease protein L3 23K (Y09598), primers for the amplification of a part of the chicken GAPDH gene were chosen based on accession number M11213. Primers and probes were synthesized by Microsynth (Balgach, Switzerland). In a first step, a conventional PCR was performed in order to test the primers. The PCR mix for the positive sample

contained 12.5 µl of RedTaq ReadyMix (Sigma, St.Louis, MO, USA), 1 µl each of forward and reverse primer (end concentration 400nM), 0.5 µl of diethylpyrocarbonate (DEPC) treated water and 10 µl of EDSV 1976 DNA. Instead of viral DNA, 10 µl of DEPC treated water was added to the PCR mix and used as negative control. The PCR was run in a thermal cycler 2720 (Applied Biosystems, Foster City, CA, USA). After one cycle of three minutes at 93°C, 35 cycles of one minute each at 93°C, 55°C and 72°C were made. The final step was carried out at 72°C for ten minutes. 10 µl of each sample and 10 µl of a 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was loaded on a 2% agarose gel. After exposition to UV light in the transilluminator, a band with 145 bp was visible in the positive sample, but not in the negative one.

3.5. DNA amplification by TaqMan technology

3.5.1. Real-time PCR for the detection of EDSV 1976 DNA

Primers described in Table 4 were used for the detection of the EDSV 1976 DNA. The reaction mixture contained 10 µl of DNA, 12.5 µl of TaqMan®Universal PCR Mastermix (2x) (Applied Biosystems, Foster City, CA, USA), 1 µl of the forward primer For_EDSV (end concentration 400 nM), 1 µl of the reverse primer Rev_EDSV (end concentration 400 nM) and 0.5 µl of the probe Probe_EDSV (end concentration 200 nM). DEPC treated water as well as an extraction control were used as negative controls. Samples were run in duplicates. The reaction was performed on Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling protocol was composed of three different stages. Stage one proceeded for two minutes at 50°C followed by stage two for 10 minutes at 95°C. Stage three consisted of 40 cycles of a denaturation step at 95°C for 15 seconds and an annealing-elongation step at 60°C for 1 minute. Results were analysed with

the program 7500 Fast System SDS Software version 1.4.0 (Applied Biosystems, Foster City, CA, USA). The threshold value using the detectors FAM and TAMRA was set at 0.03, baseline ranged from 3-15.

3.5.2. Real-time PCR for the detection of Chicken GAPDH DNA

The PCR assay for the detection of house-keeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out in the same way as EDSV 1976 PCR described under 3.5.1. Primers and probe are described in Table 4. Cycle threshold value for the detectors VIC and TAMRA was set at 0.01 and baseline ranged from 3-15.

3.6. Generation of the pCR2.1_EDSV_1976 plasmid used for standard curve analysis

The PCR product obtained with conventional PCR was cloned into a pCR2.1 vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). 4 µl of fresh PCR product was mixed with 1 µl of salt solution and 1 µl of pCR2.1 vector. A negative control with 4 µl of DEPC treated water was included. After incubation at room temperature for 30 minutes, the reaction was placed on ice. For the transformation, 2 µl of each positive and negative sample was pipetted in a vial containing chemically competent Escherichia coli cells. The content was mixed gently and incubated on ice for half an hour. Subsequently, the bacteria cells were shocked in a water bath at 42°C for 30 seconds. The tubes were placed on ice again and 250 µl SOC medium was added. The samples were incubated at 37°C for 1 hour and then streaked on a prewarmed Ampicillin containing LB agar plate (end concentration Ampicillin 100 µg / ml). The plates were incubated overnight at 37°C. Colonies from the plates were picked and used for plasmid isolation using the QIagen Plasmid Mini Kit (QIAgen, Hilden, Germany).

Obtained plasmid DNA was digested with EcoR I (NewEngland Biolabs, Ipswich, MA, USA). Two clones showing the expected restriction enzyme pattern were chosen and sent for sequencing (Microsynth, Balgach, Switzerland). One of the correct clones was further used to generate a higher yield of plasmid DNA using the PureYield™ Plasmid Maxiprep System (Promega, Madison, WI, USA). Concentration and purity of the isolated plasmid DNA was determined using the NanoDrop (Witec AG, Littau, Switzerland) and the plasmid was named pCR2.1_EDSV_1976.

3.7. Determination of the analytical sensitivity of the EDSV 1976 real-time PCR

3.7.1. Plasmid dilutions in DEPC treated water

Tenfold serial dilutions of the pCR2.1_EDSV_1976 plasmid ranging from $3x10^{10}$-$3x10^{-3}$ copies per µl were made in DEPC treated water. Dilutions ranging from $3x10^{5}$ to $3x10^{0}$ copies per reaction were used to create a standard curve. Real-time PCR amplification was carried out as described under 3.5.1. and the experiment was carried out with 4 repetitions.

3.7.2. Comparison of the EDSV 1976 real-time PCR to the conventional PCR

A conventional PCR was performed with 10 µl of the pCR2.1_EDSV_1976 plasmid dilutions ranging from $3x10^{5}$ to $3x10^{0}$ copies per reaction. The reaction mix contained 12.5 µl RedTaq ReadyMix (Sigma, St.Louis, MO, USA), 1 µl of each primer (Table 4) and 0.5 µl of DEPC treated water. The reaction was run in a thermal cycler 2720 (Applied Biosystems, Foster City, CA, USA) under the same conditions as described under 3.4. 10 µl of the obtained PCR products were loaded on a 2% agarose gel and examined under UV light. The experiments were carried out independently on 3 days.

3.7.3. Plasmid dilutions in tissue samples

Tissue samples were spiked with the pCR2.1_EDSV_1976 plasmid in order to demonstrate the ability of the newly developed assay to detect EDSV 1976 DNA in this type of material.

6 tissue samples including liver, infundibulum as well as magnum of the oviduct, trachea, kidney and lung were cut into pieces up to 25 mg and inoculated. Briefly, 20 µl of different plasmid dilutions starting from $3x10^5$ copies per µl ($6x10^6$ copies per 25 mg of tissue) to $3x10^{-1}$ copies per µl ($6x10^0$ copies per 25 mg of tissue), respectively, were added to each sample. DNA was extracted as described under 3.3.1. and examined for the presence of EDSV 1976 and GAPDH using the newly developed assay.

3.8. Determination of the analytical specificity of the EDSV 1976 real-time PCR

DNA extracted from BAdV-1, FAdV-1, GaHV-1, GaHV-2, Mycoplasma synoviae, Mycoplasma meleagridis and Mycoplasma gallisepticum as well as a dilution of the pCR2.1_EDSV_1976 plasmid ($3x10^3$ copies per reaction) was tested in order to determine the specificity of the real-time PCR. Samples were run in duplicates, and DEPC treated water was used as negative control.

3.9. Analysis of field samples

DNA was extracted from 162 tissue samples and investigated for the presence of EDSV 1976 with the newly developed assay. Furthermore, a real-time PCR to detect the house-keeping gene chicken GAPDH was performed as described above.

4. Results

4.1. Analytical sensitivity of the EDSV 1976 real-time PCR

4.1.1. Standard curve analysis

The amplification plot of the plasmid dilution series is shown in Figure 1A. The linear regression analysis demonstrated a linear correlation between the ct value and the logarithm of the amount of the plasmid in the sample over a 6-log range. The coefficient of determination R^2 for the linear regression was 0.9969 (Figure 1B). The newly developed PCR detected 30 copies in 100% of the performed experiments. In 6 out of 8 samples even 3 plasmid copies could be amplified (75%) whereas it was not possible to detect 0.3 copies (Table 5).

4.1.2. Comparison of the conventional PCR to the EDSV 1976 real-time PCR

The conventional PCR yielded clear bands up to an amount of $3x10^3$ copies per reaction, whereas weak signals were produced up to $3x10^1$ copies per reaction (Figure 2). In contrast, the real-time PCR assay was able to detect $3x10^0$ copies per reaction.

4.1.3. Sensitivity of the EDSV 1976 real-time PCR in spiked tissue samples

EDSV 1976 DNA could be detected in spiked liver, infundibulum and magnum of the oviduct, trachea, kidney and lung. The ct values ranged from 23 to 40 (Table 6).

4.2. Analytical specificity of the EDSV 1976 real-time PCR

DNA extracted from BAdV-1, FAdV-1, GaHV-1, GaHV-2 and Mycoplasma could not be detected by the newly developed real-time PCR assay. On the other hand, plasmid DNA originating from EDSV 1976 gave a positive signal (Figure 3).

4.3. Field samples

162 tissue samples were used for DNA extraction and analyzed for the presence of EDSV 1976 DNA. GAPDH DNA could be amplified from all samples, whereas no EDSV 1976 DNA was detected. Ct values for GAPDH ranged between 18 and 28.

5. Discussion

In the present work, the establishment and application of a TaqMan based real-time PCR assay for the detection and quantification of EDSV 1976 DNA is described. After having designed primers and probe for the amplification of a 145 bp fragment within the endoprotease gene, sensitivity and specificity of the newly developed assay were tested. The endoprotease gene was chosen as target sequence since it is considered as highly conserved among adenoviruses (Tong, 2002).

Current diagnostic tests for the direct detection of EDSV 1976 are based on virus isolation or polymerase chain reaction (PCR). Conventional PCR methods are described for the detection of a fragment within the EDSV 1976 hexon gene (Dhinakar Raj *et al*., 2001: Dhinakar Raj *et al*., 2003; Kumar *et al*., 2003), which represents one of the best characterized adenovirus genes (Lehmkuhl & Hobbs, 2008). Today, conventional PCR methods are often replaced by real-time PCR assays, which are less time consuming, display a lower risk of contamination and allow quantification (Callison *et al*., 2006; Luo *et al*., 2010). An important characteristic of real-time PCR assays is their higher sensitivity compared to conventional PCR methods (Lam *et al*., 2010). In this study, the conventional method yielded clear bands up to an amount of $3x10^3$ copies per reaction. Weak bands occurred up to $3x10^1$ copies. However, the real-time PCR assay was able to detect viral DNA up to $3x10^0$ copies per reaction. This indicates a higher sensitivity of the real-time approach compared to the conventional assay. In this study, we obtained an analytical sensitivity of 99.7% in the real-time. Compared to previous EDSV 1976 PCR assays our real-time PCR assay allows quantification and saving of time.

In the present study, specificity of the real-time PCR assay was analyzed using DNA from several different pathogens including viruses and bacteria. As members of the adenovirus family, Bovine adenovirus 1 and Fowl adenovirus 1 were chosen, whereas Gallid herpesvirus

1 and Gallid herpesvirus 2 represent other important avian DNA viruses. Mycoplasma synoviae, Mycoplasma gallisepticum and Mycoplasma meleagridis were included in the test, since they can be involved in reduction of egg production with misshaped eggshells. Our real-time PCR assay was able to specifically amplify EDSV 1976 DNA, all other pathogens tested yielded a negative result.

162 tissue samples from 8 laying flocks experiencing loss in egg production were screened for the presence of EDSV 1976 DNA. Since it is described in literature that the virus is spread in many organs including the female genital tract (Hess & Monreal, 2005), tissues including liver, infundibulum and magnum of the oviduct, trachea, kidney and lung were taken. EDSV 1976 DNA could not be detected in any of the investigated samples. False negative results due to incorrect DNA extraction can be excluded since the house-keeping gene coding for chicken GAPDH was detected. Inhibition by tissue components can be excluded as tissue samples spiked with various amounts of the pCR2.1_EDSV_1976 plasmid showed that the assay was able to detect EDSV 1976 in all experiments. The results of this study showing the absence of EDSV 1976 in the investigated Swiss laying flocks are in accordance with a previous serological investigation carried out between 1999 and 2003 (Hoop, personal communication, 2010). Based on the preliminary findings, it can be speculated that EDSV 1976 is not occurring in Switzerland. Nevertheless, the developed real-time PCR assay could be essential to screen Swiss laying flocks on a regular basis in future.

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7. Figures and tables

Figure 1A & 1B: DNA amplifications from standard plasmid dilutions in DEPC treated water.

The amplification plot of the performed real-time PCR assay is shown. Cycle numbers as well as the increase in fluorescence are shown on the x- respectively on the y-axis **(1A)**. The number of standard plasmid molecules per reaction is given on the x-axis, whereas ct values are presented on the y-axis. The formula for the regression curve was calculated and indicated in the figure **(1B)**.

Figure 2: Agar gel electrophoresis of conventional PCR products from standard plasmid dilutions in DEPC treated water.

M: 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA); 1: $3x10^5$ copies per reaction; 2: $3x10^4$ copies per reaction; 3: $3x10^3$ copies per reaction; 4: $3x10^2$ copies per reaction; 5: $3x10^1$ copies per reaction; 6: $3x10^0$copies per reaction; 7: Negative control DEPC treated water.

Figure 3: Amplification plot of various DNA samples tested in the EDSV 1976 real-time PCR assay

Specificity was tested using plasmid DNA from EDSV 1976 as well as DNA from BAdV-1, FAdV-1, GaHV-1, GaHV-2, Mycoplasma synoviae, Mycoplasma gallisepticum and Mycoplasma meleagridis. Cycle numbers are shown on the x-axis, the increase in fluorescence is visible on the y-axis.

Figure 1A

Figure 1B

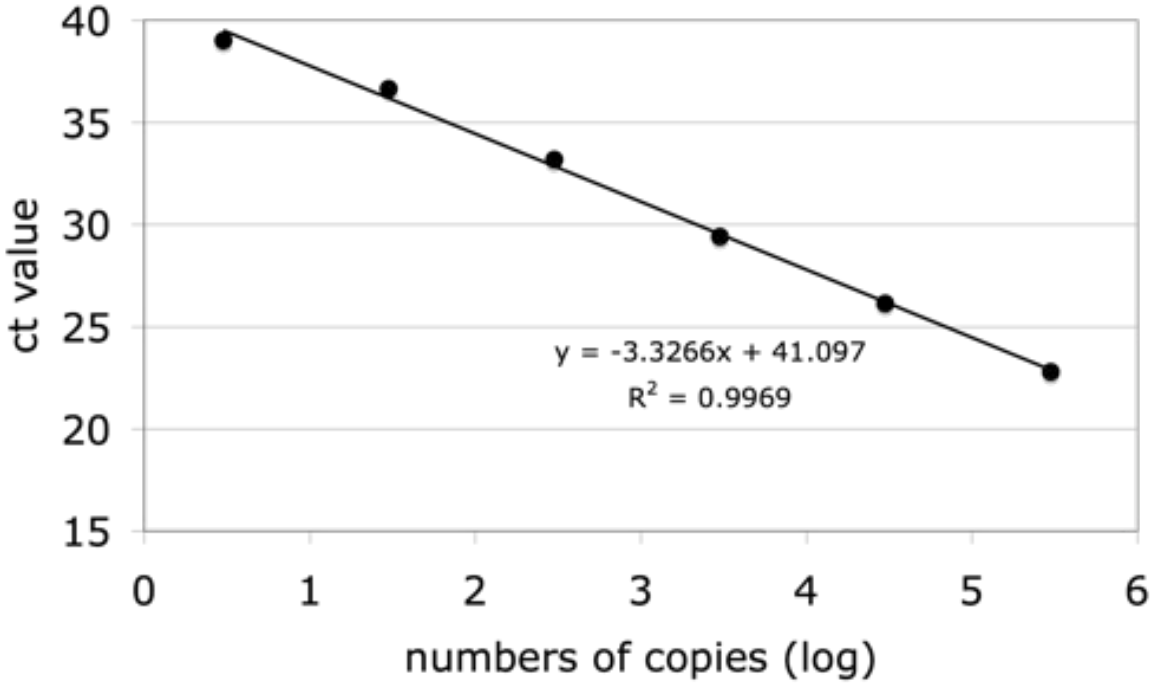

Figure 2

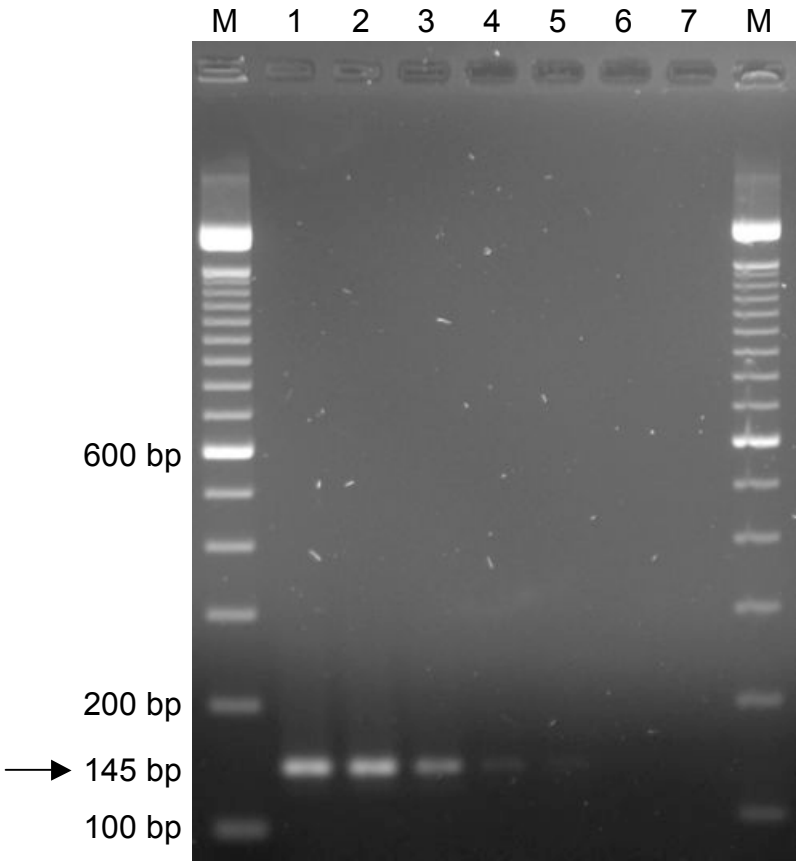

Figure 3

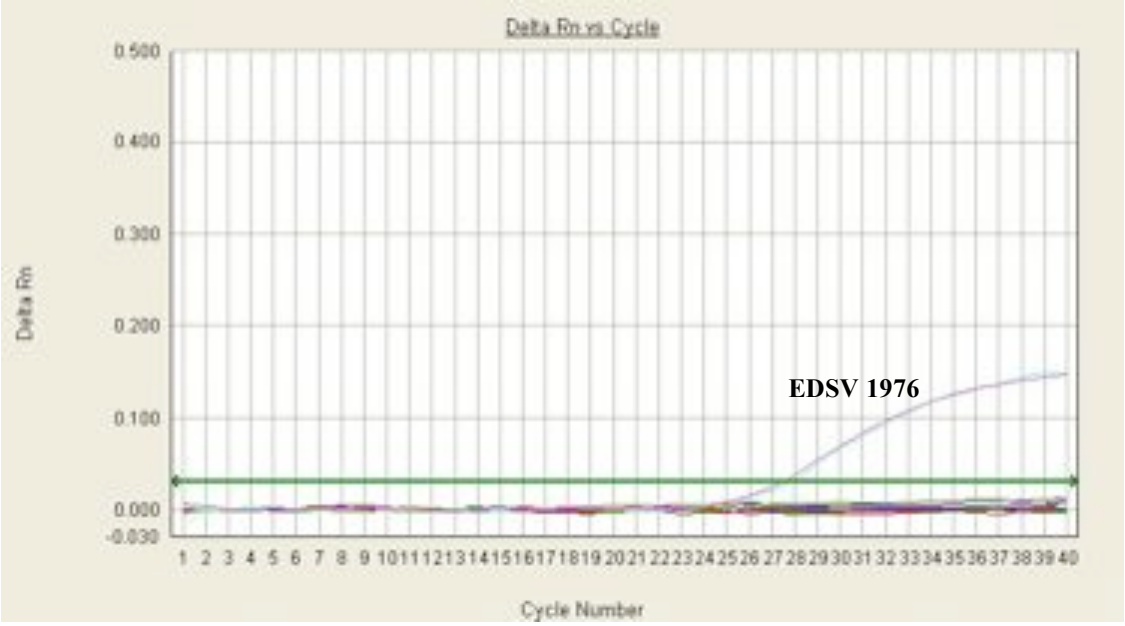
Delta Rn vs Cycle
Delta Rn
0.500
0.400
0.300
0.200
0.100
0.000
-0.030
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Cycle Number
EDSV 1976

Table 1: The family Adenoviridae.*

Family Adenoviridae	
Genus *Mastadenovirus*	Bovine adenovirus A-C Canine adenovirus Equine adenovirus A, B Human adenovirus A-G Murine adenovirus A, C Ovine adenovirus A, B Porcine adenovirus A-C Simian adenovirus A Tree shrew adenovirus
Genus *Aviadenovirus*	Falcon adenovirus A Fowl adenovirus A-E Goose adenovirus
Genus *Siadenovirus*	Frog adenovirus Raptor adenovirus A Turkey adenovirus A
Genus *Atadenovirus*	Bovine adenovirus D Ovine adenovirus D Duck adenovirus A Possum adenovirus Snake adenovirus A
Genus *Ichtadenovirus*	Sturgeon adenovirus A

* Source: ICTV virus taxonomy, 2009

Table 2: Source of field samples.

Farm	Number of animals	Breed	Age (weeks)
1	6	broiler breeders	40
2	2	layers	30
3	6	layers	59
4	3	layers	unknown
5	2	layers	25
6	5	layers	40
7	1	layers	36
8	2	layers	56

Table 3: Viral and bacterial strains.

Pathogen	Source
EDSV 1976 (DAdV-1 127)	Intervet, Boxmeer, NL
BAdV-1	Institute of Virology, Vetsuisse faculty, Zurich
FAdV-1 CELO	Department of Poultry Diseases, Zurich
GaHV-1	Department of Poultry Diseases, Zurich
GaHV-2	Department of Poultry Diseases, Zurich
Mycoplasma synoviae	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, D
Mycoplasma gallisepticum	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, D
Mycoplasma meleagridis	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, D

Table 4: Primers and probes.

Oligonucleotid	Sequence	Gene	Accession no	Amplicon size
For_EDSV	5'-ACTTGCGCTGGTAGTTGTG-3'	L3 23K	Y09598	145 bp
Rev_EDSV	5'-GCAACAGATGAGGTTTGGAAG-3'			
Probe_EDSV	5'-FAM-TGCATGGTACCTCCCCGGCT-TAMRA-3'			
For_GAPDH	5'- GGAGTCAACGGATTTGGC-3'	chicken GAPDH	M11213	135 bp
Rev_GAPDH	5'-TTTGCCAGAGAGGACGGC-3'			
Probe_GAPDH	5'-VIC-TATTGGCCGCCTGGTCACCAGG-TAMRA-3'			

Table 5: Detection limit of the EDSV 1976 real-time PCR.

Copy number	Positive / Number of runs
300'000	8/8
30'000	8/8
3'000	8/8
300	8/8
30	8/8
3	6/8
0.3	0/8

Table 6: Detection of EDSV 1976 plasmid DNA in spiked tissue samples.

Organ	Copies before extraction	Ct GAPDH	Ct EDSV 1976
liver	$6x10^6$	24/23	26/27
	$6x10^5$	24/23	31/29
	$6x10^4$	23/23	30/31
	$6x10^3$	23/23	35/34
	$6x10^2$	23/23	39/38
	$6x10^1$	23/23	undetected
	$6x10^0$	22/23	undetected
infundibulum	$6x10^6$	24/24	25/25
	$6x10^5$	23/23	27/27
	$6x10^4$	23/23	30/31
	$6x10^3$	23/23	34/34
	$6x10^2$	24/24	39/40
	$6x10^1$	24/24	undetected
	$6x10^0$	24/24	undetected
magnum	$6x10^6$	25/25	24/24
	$6x10^5$	24/24	28/29
	$6x10^4$	24/24	34/33
	$6x10^3$	24/24	undetected/38
	$6x10^2$	24/24	37/37
	$6x10^1$	24/24	undetected
	$6x10^0$	24/24	undetected
trachea	$6x10^6$	25/25	23/23
	$6x10^5$	24/24	26/27
	$6x10^4$	24/24	30/30
	$6x10^3$	25/25	34/34
	$6x10^2$	25/25	39/39
	$6x10^1$	25/24	undetected
	$6x10^0$	25/25	undetected
kidney	$6x10^6$	23/23	23/23
	$6x10^5$	23/26	26/26
	$6x10^4$	24/23	31/31
	$6x10^3$	25/34	34/35
	$6x10^2$	28/26	38/38
	$6x10^1$	25/23	undetected
	$6x10^0$	23/23	undetected
lung	$6x10^6$	23/23	23/23
	$6x10^5$	23/22	27/28
	$6x10^4$	22/22	31/31
	$6x10^3$	22/23	34/34
	$6x10^2$	22/22	38/38
	$6x10^1$	22/22	undetected
	$6x10^0$	23/23	undetected

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